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Synergy between glyphosate- and cypermethrin-based pesticides during acute exposures in tadpoles of the common South American Toad *Rhinella arenarum*

Julie Céline Brodeur *[,1], María Belén Poliserpi, María Florencia D'Andrea [1], Marisol Sánchez

Instituto de Recursos Biológicos, Centro Nacional de Investigaciones Agropecuarias (CNIA), Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Buenos Aires, Argentina

HIGHLIGHTS

- Glyphosate and cypermethrin are likely co-occur in wetlands supporting amphibians.
- Little is known regarding how glyphosate and cypermethrin interact in mixtures.
- Mixtures were significantly synergic in both combinations of products tested.
- The synergy produced toxicities 2–9 times greater than concentration addition.
- Synergies of such a great magnitude are uncommon in the literature.

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ABSTRACT

The herbicide glyphosate and the insecticide cypermethrin are key pesticides of modern management in soy and corn cultures. Although these pesticides are likely to co-occur in ephemeral ponds or aquatic systems supporting amphibian wildlife, the toxicological interactions prevailing in mixtures of these two pesticides have been little studied. The current study evaluated the toxicity of equitoxic and non-equitoxic binary mixtures of glyphosate- and cypermethrin-based pesticides to tadpoles of the common South American toad, *Rhinella arenarum*. Two different combinations of commercial products were tested: glyphosate Glifosato Atanor® + cypermethrin Xiper® and glyphosate Glifoglex® + cypermethrin Glextrin®. When tested individually, the formulations presented the following 96 h-LC50s: Glifosato Atanor® 19.4 mg ae L^{-1} and Glifoglex 72.8 mg ae L^{-1}, Xiper® 6.8 mg L^{-1} and Glextrin® 30.2 mg L^{-1}. Equitoxic and non-equitoxic mixtures were significantly synergic in both combinations of commercial products tested. The magnitude of the synergy (factor by which toxicity differed from concentration addition) was constant at around twofold for all tested proportions of the glyphosate Glifoglex® + cypermethrin Glextrin® mixture; whereas the magnitude of the synergy varied between 4 and 9 times in the glyphosate Glifosato Atanor® + cypermethrin Xiper® mixture. These results call for more research to be promptly undertaken in order to understand the mechanisms behind the synergy observed and to identify and quantify the extent of its environmental impacts.

1. Introduction

Amphibians are the most threatened and rapidly declining vertebrate group, their actual rate of extinction being approximately 200 times greater than background historic rates (Roelants et al., 2007; Hoffmann et al., 2010). Factors contributing to the amphibian crisis are diverse and include habitat loss, environmental contamination, climate change and emerging infectious diseases (Pounds et al., 2006; Sodhi et al., 2008; Egea-Serrano et al., 2012). While declines in apparently physically undisturbed habitats (the so-called 'enigmatic declines') are generally linked with disease and climate change, declines of lowland species are, for their part, mainly related to agriculture-associated habitat loss and contamination (Mann et al., 2009).

Agriculture consumes a greater proportion of land than any other human activity, the resulting extensive habitat loss

* Corresponding author. Address: Instituto de Recursos Biológicos, Centro Nacional de Investigaciones Agropecuarias (CNIA), Instituto Nacional de Tecnología Agropecuaria (INTA), 1686 Hurlingham, Buenos Aires, Argentina. Tel.: +54 11 4621 1819x106.
E-mail address: brodeur.celine@inta.gob.ar (J.C. Brodeur).
[1] Member of the "Consejo Nacional de Investigaciones Científicas y Técnicas" (CONICET), Argentina.

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negatively impacting amphibian diversity and abundance (Hecnar and M'Closkey, 1996; Bishop et al., 1999; Houlahan et al., 2000). In addition, pesticide use increased over the last decades as agriculture gradually transformed into a high-tech system for satisfying the world's growing demands for food, feed, fiber and fuel (Benbrook, 2012; Heinemann et al., 2013). In Argentina, as in the United States, the adoption of transgenic crops designed to tolerate the broad-spectrum herbicide glyphosate steadily increased over the last 17 years to reach, during the growth season of 2012–2013, a new high of 22.175 million hectares of cultivated land occupied by glyphosate-tolerant corn and soybean (66% of planted corn and 100% of planted soybean) (CONABIO, 2013). As a consequence of this increasing dependence on transgenic crops, glyphosate-based herbicides are currently the world's best selling herbicides and the backbone of modern no-till agriculture (Duke and Powles, 2008).

Glyphosate kills weeds by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme essential for the production of aromatic amino acids in plants and a few microbial species (Rubin et al., 1982). As animals obtain these aromatic amino acids through diet and lack this enzyme, glyphosate has generally been considered to pose little risk to non-target vertebrate and invertebrate species (Giesy et al., 2000; Solomon and Thompson, 2003). However, multiple laboratory studies have now shown that the commercial glyphosate formulations named Roundup®, Vision® and Glyphos® are moderately toxic to amphibian larvae with 96 h-LC50 values in the range of 1–5 mg L^{-1} (Mann and Bidwell, 1999; Lajmanovich et al., 2003; Chen et al., 2004; Edginton et al., 2004; Howe et al., 2004; Relyea, 2005; Relyea and Jones, 2009; Bernal et al., 2009a; Fuentes et al., 2011; Moore et al., 2012; Sing Yadav et al., 2013). Although these results suggest a possibility for toxic effects in the field, an increasing number of mesocosm and field studies in this range of concentrations or lower actually failed to cause significant mortality or sublethal effects in amphibian larvae, raising uncertainty regarding the toxicity of glyphosate formulations in natural systems (Wojtaszek et al., 2004; Thompson et al., 2004; Bernal et al., 2009b; Edge et al., 2011, 2012, 2013; Lanctôt et al., 2013).

In the humid pampas of Argentina, the two most common crop rotations (planting of different crops in subsequent growing seasons) are corn/full season soy and corn/wheat/short season soy. Together with 2–3 applications of glyphosate, a transgenic soyfield from the pampas region will also typically receive applications of pyrethroid or organophosphate insecticides as well as an application of fungicides such as triazoles or strobilurins. Corn crops for their parts can also receive, aside from glyphosate (in the case of transgenic plants), applications of atrazine and acetochlor herbicides, and organophosphate or pyrethroids insecticides. These pesticides can be applied alone or, more commonly, in various combinations (Rennella and Quirós, 2000; Pérez Leiva and Anastasio, 2003; Bindraban et al., 2009). For example, over the last decade, the practice of adding a small amount of the pyrethroid insecticide cypermethrin to pre- or post-emergence glyphosate applications gained large popularity amongst soybean producers from the argentine pampas as a way of eliminating potential pest insects from the field before the establishment of the crop. Although this practice is being discouraged based on agronomic proofs of its ineffectiveness for pest control (Massaro, 2010), some producers are known to still employ it.

Crop protection products applied on the fields may enter ephemeral pools or aquatic systems through drift, accidental overspray or runoff (Giesy et al., 2000; Solomon and Thompson, 2003). Extensive water quality monitoring programs from agricultural regions of North America indicate that diverse pesticide contaminants are often present at low concentrations throughout the year and that herbicides are commonly detected in 70–90% of the samples (Gilliom et al., 2006; Giroux and Pelletier, 2012). Although such extensive data sets are not available in Argentina, the widespread use of agricultural pesticides and the monitoring values available (Jergentz et al., 2005; Marino and Ronco, 2005; Peruzzo et al., 2008) make it possible to suspect a similar pattern of water contamination.

Amphibians and other aquatic or semi-aquatic animals inhabiting agricultural areas are therefore likely to be exposed to mixtures of agricultural chemicals of fluctuating composition and concentrations. Past toxicity studies involving pesticide mixtures have resulted in a full spectrum of responses in which the observed interactions depended on the chemical properties and modes of toxic action of the pesticides (Lydy et al., 2004). Although challenging, the quantification and characterization of the toxicity of specific significant mixtures is extremely important as it will eventually allow the development of methods to evaluate the risk these pose in the real world (Mumtaz, 2010).

In the Argentine pampas, glyphosate and cypermethrin are likely to occur simultaneously in ephemeral pools or aquatic systems, either as a result of the agronomic practice described above or due to the presence of neighboring crops in different stages of growth (short season soy receiving post-emergence glyphosate next to full season soy or corn receiving cypermethrin applications against pest insects). As little information exists regarding the toxicological interactions prevailing in mixtures of glyphosate and cypermethrin, the aim of the current study was to evaluate the toxicity of equitoxic and non-equitoxic binary mixtures of glyphosate and cypermethrin to tadpoles of the common South American toad, *Rhinella arenarum*.

2. Materials and methods

2.1. Animal collection and husbandry

Egg ribbons of the common South American toad, *R. arenarum* were collected in the spring when breeding events occurred in the various water bodies present within the 650 hectares of lands (34°36′24S, 58°39′56W) pertaining to the National Center for Agricultural Research (CNIA). Pesticide applications are not normally conducted on the lands belonging to the CNIA which are located in a residential sector of the suburban area surrounding Buenos Aires City. *R. arenarum* is terrestrial and congregates in large breeding groups in shallow temporary ponds that form after heavy rains. Egg ribbons were collected within 24 h of being laid and were immediately transferred to the laboratory where they were kept in a climate-controlled room at 20 ± 2 °C and 16:8 h light:dark photoperiod until reaching stage 25 (Gosner, 1960). Every time eggs were collected, 5–10 different clutches were gathered and combined in the laboratory. Tadpoles were offered boiled swiss chard *ad libitum* when they began feeding at stage 24–25.

2.2. Culture water

Well water used in holding tanks, controls and treatments exhibited the following characteristics: pH 8; chlorine <0.1 mg L^{-1}; fluor 0.97 mg L^{-1}; vanadium <0.05 mg L^{-1}; total iron <0.01 mg L^{-1}; arsenic 0.016 mg L^{-1}; lead <0.01 mg L^{-1}; calcium 18.0 mg L^{-1}; sulfates (SO_4^{2-}) 12 mg L^{-1}; nitrates (NO_3^-) 21.9 mg L^{-1}; nitrite (NO_2^-) <0.01 mg L^{-1}; ammonium (NH_4^+) 0.03 mg L^{-1}; chlorides 19.5 mg L^{-1}, conductivity 806 μs cm^{-1}; hardness 80.0 mg L^{-1}; alcalinity 373 mg L^{-1} as $CaCO_3$. All values fell within accepted guidelines for tadpole testing (ASTM, 1998).

2.3. Test substances

Two different commercial formulations of glyphosate (N-[phosphonomethyl] glycine; CAS No. 1071-83-6) were tested: Glifoglex®

and Glifosato Atanor®, which are respectively commercialized in Argentina by the firms GLEBA and ATANOR. Both formulations contain 48% of glyphosate in the form of isopropylamine salt which corresponds to 36% of glyphosate acid equivalent (ae). Cypermethrin ([Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate; CAS No. 52315-07-8), formulations tested were Glextrin® and Xiper®, which are respectively comercialized in Argentina by the firms GLEBA and ICONA. Both formulations are emulsionable concentrates and contain 25% of a mixture of cis and trans isomers of cypermethin (cis 40–50%). All four pesticide formulations tested included proprietary surfactants, additives or emulsifiers of undisclosed molecular nature.

2.4. Preparation of test solutions

Every time test solutions were changed, a stock solution was prepared for each of the pesticide being tested. The diluent used for these stock solutions was the culture water described above. The nominal concentration of the stock solutions was 3600 mg ae L^{-1} in the case of glyphosate formulations (Glifoglex® and Glifosato Atanor®) and 250 mg L^{-1} in the case of cypermethrin formulations (Glextrin® and Xiper®). Test solutions were prepared by diluting the adequate volume of stock solution in culture water.

Glyphosate concentration of stock solutions was verified through high performance liquid chromatography (HPLC)/mass spectrometry using ZORDBAX XDB-C8 (4.6 × 50 mm) and 50 mM ammonium acetate:acetronitrile, at the following gradient; (100:0) to (5:95) 0–5 min, (5:95) 5–8 min, as the mobile phase. Detection was realized with derivatization with 9-fluoromethyl chloroformate (FMOC), using quadrupole mass spectrometry, with electrospray ionization (ESI) in positive mode SIM:ion 392 *m*/*z*. Cypermethrin concentration of stock solutions was verified through gas chromatography/mass spectrometry using analytical column HP-5 ms ultra Inert 30 m × 250 μm, at the following conditions: Helium constant flow, 1 mL min^{-1}, at 60 °C for 1 min, then 40 °C min^{-1} at 170 °C, then 10–310 °C, then hold for 2 min, injection mode splitless 1 μL, inlet temperature 280 °C, transfer line temperature 280 °C. Detection was realized using quadrupole and electron ionization (EI) at SIM mode, quantifier ion 181 *m*/*z*.

2.5. Experimental protocols

2.5.1. Individual acute toxicity of glyphosate and cypermethrin formulations

Concentrations for definitive testing were determined from range-finding tests. For each of the pesticide formulations studied, final nominal concentrations tested were as follows: glifosato Atanor® 10, 15, 17.5, 20, 22.5 and 25 mg ae L^{-1}, Glifoglex® 60, 65, 70, 75, 80, 85 and 90 mg ae L^{-1}, Xiper® 2.5, 5, 7.5, 10, 12.5 mg L^{-1} and Glextrin® 10, 15, 20, 25, 30, 35, 40, 50 mg L^{-1}. The experimental design also included a control group exposed to culture water only. Six replicates were performed for every concentration tested. In every replicate, 10 tadpoles having recently reached stage 25 were placed in 70 mL of culture water with or without (controls) test chemicals. To avoid evaporation of test solution, experimental tanks consisted of two superposed 10 cm-diameter acid-washed glass petri dishes. Test solutions were entirely replaced every 24 h, and temperature was maintained between 20 ± 2 °C throughout the exposure, which lasted 96 h. Dead tadpoles were removed and survival was evaluated every day when renewing the solutions.

2.5.2. Acute toxicity of equitoxic and non-equitoxic binary mixtures of glyphosate and cypermethrin

Acute toxicity of equitoxic and non-equitoxic binary mixtures of glyphosate and cypermethrin formulations were estimated in order to determine the toxic interactions (synergy, antagonism or additivity) prevailing in these different types of mixtures. Mixture toxicity was evaluated for the following pairs of glyphosate/cypermethrin products; glifosato Atanor®/Xiper® and Glifoglex®/Glextrin®. Mixture toxicity was evaluated according to the protocols described by Warne (2003), which are based on the toxic unit (TU) concept. This concept, which was first described by Sprague (1970), arbitrarily assigns a value of 1 toxic unit to a concentration of toxicant that elicits a particular response, in the case of the present study 50% mortality (LC50).

In the case of the equitoxic mixtures, pesticide products were combined in equal proportion so as to create mixtures in which the sum of the toxic units of both components equalled: 0.125, 0.25, 0.5, 1, 2 and 4 TU. More explicitly, if the test mixture had a value of 1 TU, it contained 0.5 TU of glyphosate formulation (1/2 of the LC50) and 0.5 TU of cypermethrin formulation (1/2 of the LC50), i.e. the combined total toxicity was 1 TU. Tadpoles were exposed to these six mixtures for 96 h according to the experimental procedures described above for single pesticide exposures. A control group exposed to culture water only was also included in the experiment. Based on the mortality values obtained, a TU-response relationship was developed and used to calculate the TU value of the mixture that caused 50% of mortality (TU50).

For non-equitoxic mixtures, four series of experiments were conducted; each of the series including six treatment groups. In the first two series, the concentration of glyphosate formulation was fixed at 0.33 TU (series 1) or 066 TU (series 2) in all the treatment groups while the concentration of cypermethrin formulation was 0.01, 0.05, 0.1, 0.5, 1 or 2 TU. Conversely, in series 3 and 4, cypermethrin formulation concentration was set at 0.33 and 0.66 TU, respectively, while the concentration of glyphosate formulation was either 0.01, 0.05, 0.1, 0.5, 1 or 2 TU. Tadpoles were exposed to these twenty-four mixtures for 96 h according to the experimental procedures described above for single pesticide exposures. A control group exposed to culture water only was also included in the experiment. Based on the mortality values obtained, a TU-response relationship was developed for each of the four series and used to calculate a TU50 in each case.

2.6. Data analysis

The concentrations of pesticides and TU of the mixtures resulting in the mortality of 50% of tadpoles were calculated by fitting a four-parameter logistic regression equation to the survival data using the GraphPad Prism software version 3.02. The values of LC50 obtained for the commercial formulations of either glyphosate or cypermethrin were compared by a *t*-test as were the values of TU50 calculated for the equitoxic mixtures. All *t*-test were conducted using SigmaStat 3.11 statistical software (SPSS, Chicago, IL, USA).

3. Results

3.1. Pesticide concentrations in stock solutions

Actual concentrations of stock solutions did not deviate from the nominal concentrations and averaged (mean ± standard error, $n = 4$), 3582 ± 248 mg ae L^{-1} in the case of glyphosate and 231 ± 10.9 mg L^{-1} for cypermethrin.

3.2. Individual acute toxicity of glyphosate and cypermethrin formulations

Survival of tadpoles in the control groups was between 95% and 100%. Concentrations of glyphosate and cypermethrin causing the mortality of 50% of tadpoles after 96 h of exposure are shown in Table 1. Values obtained for the two commercial formulations tested were significantly different for both glyphosate and

cypermethrin. In both cases, the products from the firm Gleba (Glifoglex® and Glextrin®) were considerably less toxic than the other products; the value of LC50 obtained for Glifoglex® being 3.8 times higher than that of Glifosato ATANOR® and the LC50 of Glextrin® being 4.4 times superior to that of Xiper® (Table 1). The wide variation in toxicity observed amongst formulations of the same pesticide makes it impossible to declare either cypermethrin or glyphosate formulations as being most toxic since the following alternate arrangement is obtained when ranking the four pesticide formulations from higher to lower toxicity: cypermethrin Xiper® > glyphosate Glifosato ATANOR® > cypermethrin Glextrin® > glyphosate Glifoglex®.

3.3. Acute toxicity of equitoxic and non-equitoxic binary mixtures of glyphosate and cypermethrin

Survival of tadpoles in the control groups was between 98% and 100%. Toxic units of the equitoxic mixtures of glyphosate and cypermethrin that generated the mortality of 50% of the tadpoles are shown in Table 2. These values were not significantly different and corresponded to 0.58 and 0.47 TU in the case of the Glifosato ATANOR® + Xiper® and Glifoglex® + Glextrin® mixtures, respectively. The fact that both TU50 are lower than 1 indicates that glyphosate and cypermethrin formulations present greater than additive interactions in both mixtures.

The isobologram shown in Fig. 1 illustrates results obtained for the four series of non-equitoxic mixtures. The diagonal isobole linking the values on the *y* and *x* axes with values of 1 TU is the line of concentration addition (Fig. 1). The fact that all combinations of glyphosate and cypermethrin causing 50% of mortality lie below and to the left of the additivity line is suggestive of synergic interactions in all non-equitoxic mixtures tested (Warne, 2003). Table 3 shows the factors by which the different types of non-equitoxic mixtures differed from concentration addition. This table highlights the fact that synergism was 1.6–3.9 times more important in the mixture of Glifosato ATANOR® + Xiper® than in the mixture of Glifoglex® + Glextrin®. Another difference between the two non-equitoxic mixtures lies in the fact that the factor of deviation from concentration addition was constant at about 2 in the case of the Glifoglex® + Glextrin® mixture, whereas it varied from about 4 to 7–9 in the mixture of Glifosato ATANOR® + Xiper®. In this last mixture, deviation from concentration addition was lower when either glyphosate or cypermethrin concentrations were maintained stable at 0.33 TU while testing, whereas deviation from concentration addition climbed to a factor of 7 or 9 if either one of the two pesticide formulations was kept at 0.66 TU during testing.

4. Discussion

In the environment, organisms are most commonly exposed to mixtures of chemicals rather than single compounds. This is especially true for amphibians living in agricultural areas as different agrochemicals are commonly applied simultaneously in nearby lots or to a given crop. The current study examined the toxicity and interactions prevailing in equitoxic and non-equitoxic mixtures of commercial formulations of glyphosate and cypermethrin. The study of non-equitoxic mixtures provides a much fuller account of the toxicity of the mixture than when only the equitoxic mixture is examined. In addition, this approach is more environmentally realistic, as it is highly unlikely that chemicals in a mixture will occur in an equitoxic form in the environment (Warne, 2003).

Before studying the mixtures, it was essential to precisely quantify the individual acute toxicity of every one of the pesticide formulations tested. Results obtained demonstrated the existence of a rather large variation in the toxicity of the two formulations tested for both cypermethrin and glyphosate products (Table 1). This finding is consistent with existing literature, as acute toxicities of glyphosate and cypermethrin are often found to depend largely on the commercial formulation tested (Soderlund, 1991; Mann et al., 2009; Wagner et al., 2013).

In the case of glyphosate formulations, toxicity depends greatly on the surfactant employed in the formulation (Mann et al., 2009; Wagner et al., 2013). Laboratory studies demonstrated that the

Table 1
Concentrations of glyphosate and cypermethrin causing the mortality of 50% of *Rhinella arenarum* tadpoles (LC50) after 96 h of exposure. Confidence intervals 95% (CI) are indicated in parenthesis.

	96 h – LC50
Glyphosate	
Glifosato ATANOR®	19.4 mg ae L^{-1} (18.9–19.9 mg ae L^{-1})
Glifoglex®	72.8 mg ae L^{-1} (71.6–73.9 mg ae L^{-1})
Cypermethrin	
Xiper®	6.8 mg L^{-1} (6.1–7.5 mg L^{-1})
Glextrin®	30.2 mg L^{-1} (27.3–33.3 mg L^{-1})

Table 2
Toxic units (TU) of equitoxic mixtures of glyphosate and cypermethrin causing the mortality of 50% of *Rhinella arenarum* tadpoles (LC50) after 96 h of exposure. Confidence intervals 95% (CI) are indicated in parenthesis.

Equitoxic mixtures	96 h – LC50
Glifosato ATANOR® + Xiper®	0.58 TU (0.51–0.66 TU)
Glifoglex® + Glextrin®	0.47 TU (0.41–0.54 TU)

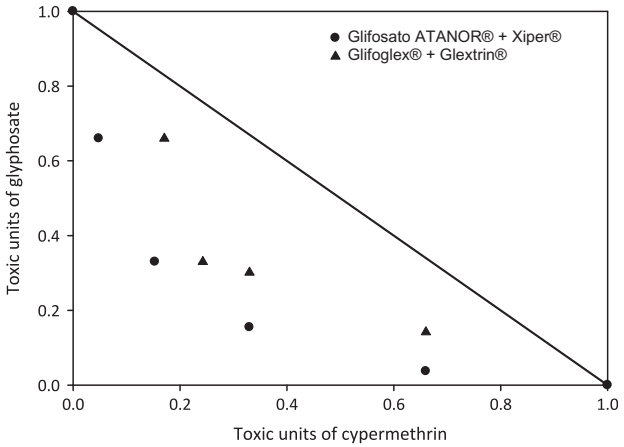

Fig. 1. Isobologram illustrating the composition of the non-equitoxic mixtures causing 50% mortality in *Rhinella arenarum* tadpoles (LC50) after 96 h of exposure.

Table 3
Factor by which the different types of non-equitoxic mixtures tested differed from concentration addition.

	Glifosato ATANOR® + Xiper®	Glifoglex® + Glextrin®
Cypermethrin 0.33 TU	4.46	2.22
Cypermethrin 0.66 TU	9.19	2.40
Glyphosate 0.33 TU	4.38	2.76
Glyphosate 0.66 TU	7.08	1.99

toxicity of Roundup Original® (the first glyphosate-based herbicide to be commercialized), is largely due to the polyethoxylated tallow amine surfactant contained in the mixture (Folmar et al., 1979; Mann and Bidwell, 1999; Edginton et al., 2004; Moore et al., 2012). With the removal of patent protection for glyphosate in 2000, many new glyphosate-base herbicides have entered the market (Howe et al., 2004). Each of these products has a slightly different chemistry and surfactant mixture, and some are considerably less acutely toxic than the original Roundup® formulation (Lajmanovich et al., 2010). This was the case for both glyphosate formulations tested in the current study, which presented LC50 values 20–36 times greater (Table 1) than the values of 1–5 mg ae L^{-1} normally calculated in tadpoles for Roundup Original® (Mann and Bidwell, 1999; Chen et al., 2004; Edginton et al., 2004; Howe et al., 2004; Relyea, 2005; Relyea and Jones, 2009; Bernal et al., 2009a; Fuentes et al., 2011; Moore et al., 2012; Sing Yadav et al., 2013). The LC-50 value of 72.8 mg ae L^{-1} calculated for Glifoglex® in the current study (Table 1) is consistent with the value of 73.77 mg ae L^{-1} previously presented by Lajmanovich et al. (2010) for the same anuran specie and glyphosate formulation.

While the toxicity of cypermethrin-based insecticides similarly depends on the carriers and emulsifiers included in the formulation, the toxicity of these products is furthermore influenced by the ratio of cis- and trans-cypermethrin isomers present in the mixture (Glickman and Casida, 1982). The cypermethrin molecule contains three chiral centres, resulting in a total of eight possible stereoisomers: four cis- and four trans-isomers. Every isomer possesses its own chemical and biological properties as well as toxicity, the cis-isomers being generally more toxic than the trans-isomers (Bradbury and Coats, 1989). With LC50s of 6.8 and 30.2 mg L^{-1} (Table 1), both cypermetrin formulations tested in the current study are comparatively less toxic then other products as most previously published data regarding the acute toxicity of cypermethrin in tadpoles are in the range of 5–500 μg L^{-1} (Izaguirre et al., 2000; Casco et al., 2006; Saha and Kaviraj, 2008; Agostini et al., 2010; David et al., 2012).

Experiments conducted in the present study on both equitoxic and non-equitoxic mixtures of glyphosate and cypermetrin suggested the presence of synergism in both pairs of commercial formulations tested (Table 2 and Fig. 1). These results are, to our knowledge, the first to clearly prove the presence of such a synergistic toxicological interaction between these two pesticides. In previous studies, azole fungicides were shown to enhance the toxic effects of pyrethroid insecticides in a number of living organisms (Chalvet-Monfray et al., 1996; Cedergreen et al., 2006; Bjergager et al., 2012), while organophosphate insecticides were found to present synergism with several other pesticides, including triazine herbicides (Woods et al., 2002; Trimble and Lydy, 2006; Pérez et al., 2013). Such synergistic interactions represent, however, a minority of the cases as evidence shows that 70–80% of chemical mixtures have additive toxicity, 10–15% have antagonistic toxicity and 10–15% have synergistic toxicity (Warne, 2003).

The fact that the magnitude of the deviation from concentration addition was 1.6 and 3.9 times greater in the mixture of Glifosato ATANOR® + Xiper® than in the mixture of Glifoglex® + Glextrin® (Table 3) is a clear indication that components of the formulation such as surfactants, solvents or emulsifiers play a role in the results observed. Indeed, if the interaction only depended on the cypermethrin and glyphosate molecules, then the magnitude of the effect would be similar in both mixtures. Interestingly, the formulations that presented the greatest synergy when in mixture were also the ones that were most toxic when tested individually (Glifosato ATANOR® and Xiper®). This observation also points out to a role of the formulation components in the interaction and is somewhat worrisome from an environmental point of view as synergies of particularly high magnitudes were observed in the current study even though pesticide formulations used were amongst the least toxic of those described in the literature. Overall, these findings highlight the difficulty of predicting the toxicity of mixtures of formulated pesticides as these are themselves mixtures which include, in addition to the active ingredient, proprietary surfactants, and additives or emulsifiers of undisclosed molecular nature. As the exact composition of the formulation will vary between different commercial products, so will the exact magnitude or nature of the interaction observed between two specific formulations.

Many studies reported that only 5% of the mixtures present a synergy more than twofold greater than concentration addition while 1% of mixtures have toxicity values that differ from concentration addition by a factor of more than 5 (Deneer, 2000; Warne, 2003; Belden et al., 2007; Cedergreen et al., 2008). The synergy observed in the current study between glyphosate- and cypermethrin-based pesticides is therefore of great interest as it produced toxicities 2–9 times greater than those predicted by concentration addition, a phenomenon uncommonly seen in the literature. Further studies should therefore be designed to investigate both the biochemical and physiological basis of the synergy as well as its potential environmental consequences.

It would be especially important for risk assessment purposes to determine whether the synergy observed here in acute exposures is also present in chronic exposure to low concentrations. In any case, the high potential for toxicological synergy highlighted in the present study coupled to the demonstrated agronomic ineffectiveness of the practice of adding a small amount of the cypermethrin to pre- or post-emergence glyphosate applications (Massaro, 2010) should be sufficient to impulse the establishment of educational programs and regulatory measures aimed at dissuading the use of this practice.

In conclusion, the current study demonstrates that glyphosate- and cypermethrin-based pesticides exhibit synergic interactions during acute exposures in tadpoles of the common South American toad, *R. arenarum*. Given the widespread use glyphosate and cypermethrin in soy and corn cultures, it is likely that these two pesticides will occasionally co-occur in ephemeral ponds or aquatic systems supporting amphibian wildlife. In view of the current amphibian declines and their close association to agriculture, the findings obtain in the current study urge for more research to be promptly undertaken in order to understand the mechanisms behind the synergy observed and to identify and quantify the extent of its environmental impacts.

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